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ABSTRACT: The potential ecological impacts of anaerobic degradation of vegetable oil on freshwater sediments were investigated. Sediment toxicity was evaluated using two regulatory biotests: the Microtox® Solid Phase Test and an amphipod (*Hyalella azteca*) bioassay. The results of the Microtox test showed that the toxicity of the vegetable-oil-contaminated sediments (about 17–33 g oil/kg dry sediments) increased after 2 weeks incubation and then decreased to near background levels after incubation for 8 weeks under anaerobic conditions. The amphipod toxicity bioassay showed that the toxicity of fresh contaminated sediments decreased over time and returned to background levels within 8 weeks. These results suggest that the impact of vegetable oils on organisms within sediments may be limited. To account for the significance of environmental conditions, additional studies over a wide range of incubation conditions (e.g., temperature, nutrient concentration) and other test organisms at various trophic levels are recommended for both acute and chronic toxicity assessment. © 2007 Wiley Periodicals, Inc. Environ Toxicol 22: 1–8, 2007.

Keywords: toxicity; Microtox® solid phase test; *Hyalella azteca* amphipod bioassay; vegetable oil; anaerobic biodegradation; freshwater sediments

INTRODUCTION

The global trade in vegetable oils has been growing steadily over the past decade. More than 110 million metric tons of vegetable oils were produced during the 2004/2005 production year in the United States (Ash and Dohlman, 2006), and oilseeds have replaced wheat as western Canada's largest source of revenue for grain farms since 1988, with canola oil accounting for 82% of vegetable oil production (Gunstone, 2004). Demand for vegetable oils has resulted in a sharp increase in total volume and the number of shipments of vegetable oil. The accidental release of nonpetroleum substances, such as vegetable oils and animal fats, into inland-and-offshore waters is relatively common in North America (EPA, 1997). Although vegetable oils do not contain the acutely toxic compounds that are present in crude oil and refined petroleum products (e.g., aromatic hydrocarbons), vegetable oil spills can have harmful effects

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on sensitive aquatic organisms and ecosystems (Crump-Wiesner and Jennings, 1975; McKelvey et al., 1980; Mudge, 1995). The most common effects include oiling of aquatic animals, depletion of dissolved oxygen from the water column, and mortality of sessile animals (Crump-Wiesner and Jennings, 1975; Mudge et al., 1993; Mudge, 1995; Calanog et al., 1999; Pereira et al., 2002, 2003b).

Because most of the harmful effects of vegetable oil spills occur when the oil is floating on the water surface or suspended in the water column, the detrimental effects of vegetable oil spills can be reduced considerably by physically removing the slick from the water surface (Rigger, 1997). Mechanical responses such as booming and skimming techniques can be effective, but they are slow, labor intensive, and expensive. In response to these limitations, a bioremediation countermeasure based on sedimentation followed by anaerobic biodegradation has been proposed as a rapid and effective technique to minimize the harmful effects of vegetable oil spills in aquatic environments (Li et al., 2001; Wincele et al., 2004). Previous work has demonstrated the feasibility of removing floating vegetable oil from the water surface by using clay to promote sedimentation as dense oil-mineral-aggregates (Wincele et al., 2004). In addition, the anaerobic biodegradability of vegetable oil has been demonstrated in freshwater sediments (Li et al., 2001, 2005; Li and Wrenn, 2004).

Although clay can effectively transfer vegetable oil from the water to sediments where anaerobic degradation of vegetable oil can occur, the metabolic intermediates of degradation (especially free long-chain fatty acids), may be toxic to microorganisms due to their ability to disrupt cell membranes and membrane-dependent processes (Galbraith and Miller, 1973; Cherrington et al., 1991). Free long-chain fatty acids have been reported to be toxic to a wide variety of microorganisms in anaerobic digestion systems (Hanaki et al., 1981; Koster and Cramer, 1987; Rinzema et al., 1994; Hwu et al., 1996; Lalman and Bagley, 2001; Pereira et al., 2001, 2003a). Transient sediment toxicity was also observed during the anaerobic biodegradation of vegetable oil in freshwater sediments (Li and Wrenn, 2004). Thus, the potential harmful effects of free fatty acids on benthic ecosystems are cause for concern.

The objective of this study was to evaluate the toxicity produced by the anaerobic biodegradation of vegetable oil in freshwater sediments. In particular, the impact of oil concentration and time on sediment toxicity was investigated. The toxicity of the oil-treated-sediments was assessed with two regulatory biotests: the Microtox® Solid Phase Test (SPT), which can be used to determine the acute toxicity of sediment, and an amphipod (*Hyalella azteca*) bioassay, which measures the lethal and sublethal toxicity of sediments. The Microtox® test has been used widely over the past two decades for toxicity assessment of soil and sediments (Cook and Wells, 1996; Bombardier and Bermingham, 1999; Doberty, 2001; Lee et al., 2003b), and the Microtox® SPT has been used successfully to evaluate the efficacy of oil spill remediation strategies (Lee et al., 1995, 1997, 2003b). Likewise, amphipod survival (e.g., *Hyalella azteca, Eohaustorius esturius*) has been often used to evaluate sediment toxicity in recent years (Ingersoll et al., 1994, 1996, 1998; Kemble et al., 1994, 1998, 1999; Bhattacharyya et al., 2003; Blaise et al., 2004). In comparison to other biotests, such as echinoid fertilization, microinvertebrate embryo-larval toxicity, and induction of mixed function oxygenase, the Microtox® SPT and amphipod tests appeared among the most sensitive assays for evaluating toxicity of sediments and interstitial waters during oil spill impact assessment (Lee et al., 2003a).

MATERIALS AND METHODS

Oil Degrading Microcosms

Sediments for toxicity tests were prepared in anaerobic oil-degrading microcosms constructed using 2.8-L Erlenmeyer flasks in an anaerobic chamber filled with nitrogen. Each microcosm contained 300 g (dry wet) of freshwater sediments (collected from Folly Lake, Westmorland County, NB, Canada), 300 g montmorillonite K10 clay (Sigma-Aldrich, St. Louis, MO, USA), and nutrient medium. Folly Lake sediments had a moisture content of 22.4%, and the dry solid consisted of 94% sand, 5% silt, and 1% clay, with an organic carbon content of 0.44%. Chemical analysis verified the absence of toxicants, such as aromatic hydrocarbons and heavy metals, in the background sediments (Table I). The sediment was sieved (1 mm) to remove indigenous organisms and large particles and thoroughly homogenized prior to construction of the microcosms. The composition of the mineral salts medium was (mg/L): K_2HPO_4 (1206), KH_2PO_4 (420), NH_4Cl (10), H_3BO_3 (0.057), Na_2MoO_4 (0.06), $CuSO_4{\cdot}5H_2O$ (0.05), $CaCl_2{\cdot}2H_2O$ (0.53), $CoSO_4{\cdot}\ 7H_2O$ (0.99), EDTA (11.6), $FeCl_3$ (0.3), $MnSO_4{\cdot}2H_2O$ (2.2), $MgSO_4{\cdot}7H_2O$ (15), $ZnSO_4{\cdot}7H_2O$ (0.9), $CaCl_2$ (147), KCl (336), NaCl (305), $MgCl_2$ (3150), and yeast extract (500). The pH of the medium was adjusted to 7.0, and it was sterilized by autoclaving. The nutrient medium was sparged with filtered nitrogen while cooling to remove dissolved oxygen. Montmorillonite clay was adjusted to neutral pH with sodium hydroxide solution and then washed thoroughly with deionized water to remove sulfate. In addition, 10 or 20 g of canola oil (Hunt-Wesson, Inc., Fullerton, CA, USA) was included in the experimental microcosms (except the control). The nominal oil loadings in the sediments were 0, 17, or 33 g/kg for the control, low-concentration, and high-concentration microcosms, respectively.

All treatments were conducted in independent triplicate microcosms incubated without mixing at room temperature ($22 \pm 1^{\circ}C$) in the dark. The volume of methane produced in the microcosms was measured by displacement of 0.1 M

TABLE I. Chemical analysis of the Folly Lake, NB, reference sediments[a]

Parameter	Concentration
Petroleum hydrocarbons	
Naphthalene	ND[b]
Acenaphthylene	ND
Acenaphthene	ND
Fluorene	ND
Phenanthrene	0.01
Anthracene	ND
Fluoranthene	0.02
Pyrene	0.02
Benz(a)anthracene	ND
Chrysene/Triphenylene	0.01
Benzo(b + k)fluoranthene	0.01
Benzo(e)pyrene	0.01
Benzo(a)pyrene	0.01
Indenopyrene	0.01
Benzo(ghi)perylene	0.01
Dibenz(a,h)anthracene	ND
Benzene	ND
Toluene	ND
Ethylbenzene	ND
Xylene	ND
TPH Purgeables (C6-C10)	ND
TPH Extractables (C10-C21)	ND
TPH Extractables(C21-C32)	ND
PCBs	ND
Metals	
Arsenic	<1
Cadmium	<0.1
Chromium	6
Cobalt	1.6
Copper	4
Lead	6.8
Mercury	<0.01
Nickel	4
Silver	<0.1
Vanadium	12
Zinc	13

[a] All units are μg/g dry sediment.
[b] ND, not detected.

sodium hydroxide from a second 0.5-L flask, which was connected through a spigot to a 100 mL graduated cylinder. After 0, 2, and 8 weeks of incubation, three replicate microcosms per treatment (0, 17, 33 g oil/kg sediment) were sacrificed for measurement of residual oil mass and sediment toxicity. The concentration of residual oil was determined by extraction with dichloromethane (EPA, 1996) followed by gravimetric measurement of the extracted oil by evaporating 1 mL aliquots of the extract to a constant mass (NETAC, 1993). For quality assurance and quality control purposes, 10% of the analyses were duplicated. The relative percent differences of these duplicates were required to be 10% or less. Two blank dishes were carried through the whole process to determine the effect of humidity on the mass determination. The accuracy and precision of the assay were checked by analyzing two replicates of a sample containing a known concentration of standard oil. Typical recoveries of spiked oil were 90% $\pm$ 10%.

Sediment samples for toxicity analysis were collected in 120 mL glass jars with nitrogen-filled headspaces and stored frozen until analysis. Toxicity of the oil-contaminated sediments was measured with the Microtox® Solid-Phase Test and an amphipod bioassay.

Toxicity Assays

The Microtox® SPT is based on the suppression of bioluminescence of the marine bacterium *Vibrio fischeri* following exposure to toxicants (Microbics Corporation, 1992) and was conducted following a previously described protocol (Lee et al., 2003b). Data were analyzed graphically with MicrotoxOmni software (SDI, Newark, DE, USA). The Microtox® SPT toxicity was expressed as EC_{50} (mg dry weight per mL), which is the effective concentration that reduced bioluminescence by 50% relative to the controls. The dry mass of sediments in the slurry was determined by drying samples at 105°C for at least 1 h (APHA, 1992).

The freshwater amphipod bioassay used *Hyalella azteca* to assess the toxicity of the sediments to microinvertebrates. The test measures the effects of sediment samples on survival and growth (as mean dry weight at the end of the test). Ten laboratory-cultured *H. azteca* neonates (2- to 9-day-old) were transferred to each of five replicated test jars (containing 100 mL portions of homogenized test sediments and 175 mL of dilution water) that were maintained under controlled experimental conditions (e.g., aeration, photoperiod, feeding regime) for an exposure of 14 days at 23 $\pm$ 1°C (Environment Canada, 1997). After 14 days, the jar contents were sieved (0.5-mm mesh), and the specimens were inspected under a dissecting microscope for 5–10 s to determine mortality. Immobile and missing animals were recorded as dead. The mean percentage survival of amphipods exposed to each treatment was compared to the mean percentage survival of amphipods exposed to the control sediment to determine if the treatments caused a significant decrease in organism survival. Surviving *H. azteca* from each exposure beaker were pooled, dried (60°C, 24 h), cooled (1 h), and weighed to measure growth. The average dry weight of surviving animals from a treatment was compared to that of the control animals to determine if exposure to treated sediment caused a significant decrease in growth. A 96-h reference toxicant test using copper chloride was conducted in water-only exposures to ensure the maintenance of normal operating conditions and the normal sensitivity of the amphipod population used in the test. A test was considered invalid if the survival of test organisms in the control sediments after 2 weeks was less than 80% or the average dry weight of the replicate groups of control

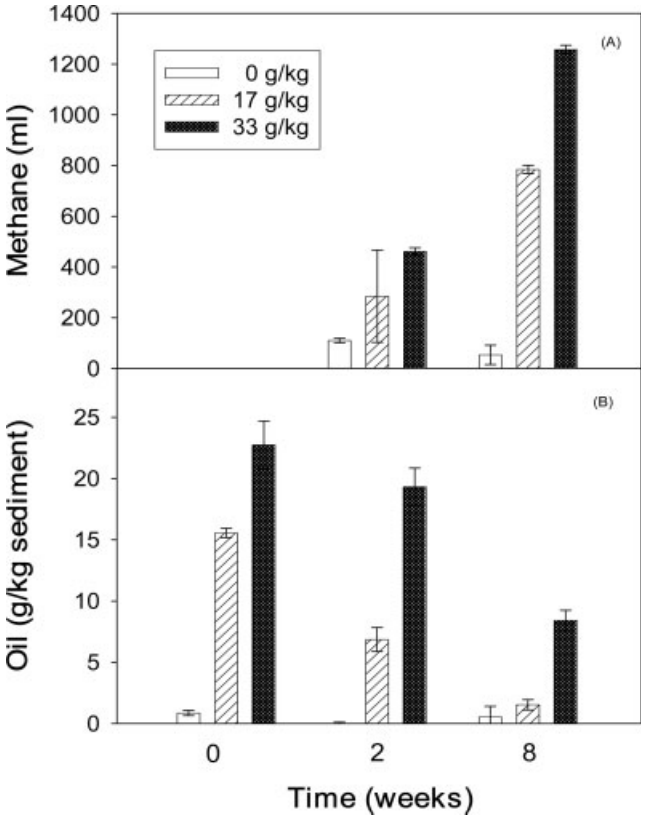

Fig. 1. Anaerobic biodegradation of canola oil in freshwater sediments: (A) methane production and (B) residual oil concentration.

organisms was less than 0.1 mg per animal (Environment Canada, 1997).

Sigma Stat 2.0 (SPSS Science, Chicago, IL, USA) was used to perform an analysis of variance on each dataset to determine the statistical significance of any differences observed between treatments over time.

RESULTS AND DISCUSSION

Methane production from the anaerobic biodegradation of canola oil in freshwater sediments was observed after 2 weeks and increased significantly after 8 weeks. The microcosms containing a higher initial concentration of canola oil yielded a significantly higher amount of methane [Fig. 1(a)], and the mass of residual oil declined correspondingly with the anaerobic oil biodegradation [Fig. 1(b)]. Although it is biodegradable, anaerobic metabolism of vegetable oil may produce intermediates that are inhibitory to aceticlastic methanogens and hydrogen-producing acetogens (Hanaki et al., 1981; Koster and Cramer, 1987; Hwu et al., 1996; Lalman and Bagley, 2001). Such inhibition was observed during anaerobic degradation of vegetable oil in freshwater sediments (Li et al., 2001, 2005). Vegetable oils and animal fats have been reported to be phytotoxic (Forster, 1992; Sampedro et al., 2005) and to exert other toxic effects on fish (Pustowka et al., 2000) in aquatic ecosystems. Furthermore, vegetable oil spills were observed to cause lethal toxicity to mussels, and dehydration and formation of intestinal lesions to birds and aquatic mammals (Mudge, 1995). Concern over potential toxic effects of sediments contaminated with vegetable oil motivated the current research. In this study, sediment toxicity during anaerobic vegetable oil biodegradation was evaluated using two regulatory biotests: the Microtox® SPT, which measures the toxicity of sediments to a bioluminescent bacterium, and an amphipod bioassay, which tests the toxicity of whole sediments to *Hyalella azteca*, a representative benthic invertebrate.

The toxicity of sediments from vegetable-oil-amended microcosms was compared to that of unoiled controls using the Microtox® SPT to determine whether canola oil or its metabolic intermediates are inhibitory in a standard microbial toxicity test. The sediment concentrations that inhibited bioluminescence by 50% (EC_{50}) are shown in Figure 2 for three initial oil concentrations and the three incubation times; higher values of EC_{50} correspond to lower sediment toxicity. The average EC_{50} values (25.5 ± 1.8 mg/mL) for the unoiled freshwater sediments were statistically indistinguishable at all incubation times ($P = 0.88$), indicating that the control sediments were stable and nontoxic. Amendment of sediments with 17 g canola oil per kg sediments decreased the EC_{50} (i.e., increased the sediment toxicity) by 15% ± 6% and 42% ± 14% relative to the controls after incubation for 0 and 2 weeks, respectively. When the oil-loading was doubled to 33 g oil/kg sediments, the EC_{50} was reduced by 43% ± 4% and 89% ± 0.5% to 14.9 and

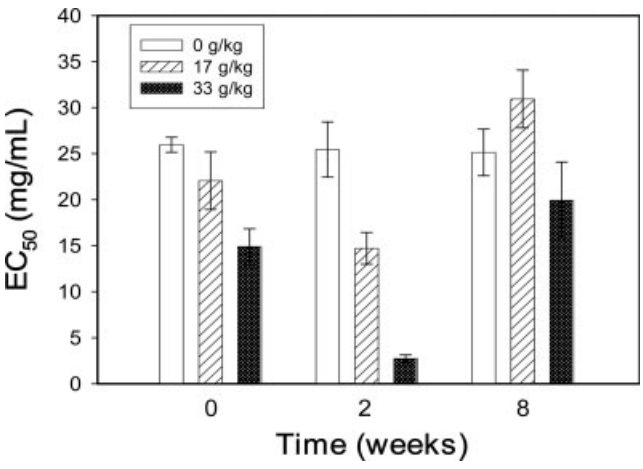

Fig. 2. Sediment concentration that causes 50% reduction in bioluminescence, EC_{50}, by *V. fischeri* in the Microtox® SPT after anaerobic biodegradation of canola oil in sediment microcosms for 0, 2, and 8 weeks. Error bars represent one standard deviation of the EC_{50} measured in independent triplicate microcosms.

2.7 mg/mL^{-1} after incubation for 0 and 2 weeks, respectively. After 8 weeks incubation, however, the average EC_{50} for sediments that were contaminated with initial oil concentrations of 17 and 33 g/kg^{-1} increased to 123% ± 14% and 79% ± 24% of the value of the unoiled control sediments, respectively, suggesting that the inhibition of oil-contaminated sediments was significantly ($P < 0.01$) reduced by anaerobic degradation of the oil. Interestingly, the sample taken from the oiled sediments at 17 g/kg had a higher EC_{50} than the unoiled controlled sediment samples, presumably due to stimulation of the test organisms by the metabolites of anaerobic biodegradation of canola oil, which could have served as an energy source.

The sediment EC_{50} values were significantly ($P < 0.01$) reduced in the presence of vegetable oil for incubation times of 0 and 2 weeks relative to the no-oil controls. While trends were observed, in terms of Canadian regulatory compliance, based on the threshold toxicity concentration (1 mg/mL^{-1}) established in the Ocean-Dumping Guidelines of Environmental Canada (Tay et al., 1997), none of the sediments would be deemed toxic at any point during the anaerobic incubation.

The toxicity of oil-contaminated sediments were also measured using an amphipod (*Hyalella azteca*) bioassay after different periods of anaerobic incubation. The toxicity of sediments to *H. azteca* can be measured based on survival and growth (in weight or length) or reproduction. In this study, the endpoints of survival and growth in weight were used to evaluate the toxicity of oil-contaminated sediments after different extents of oil degradation.

Survival of the animals exposed to the laboratory control sediments ranged from 97 to 100%, and the average mass of these control animals increased from 0.13 to 0.27 mg per specimen (Table II). These quality control data meet the acceptability criteria for the amphipod toxicity assay (Environment Canada, 1997).

The lethal toxicity of the oil-contaminated sediments was compared to the unoiled control sediments based on survival of *H. azteca* after 14 days exposure to sediments containing different oil loadings and subjected to anaerobic biodegradation for different incubation times. The average survival for oil loading and incubation time is shown in Figure 3(a). The average survival for animals exposed to unoiled sediments ranged from 93 to 100% at all three incubation times. These differences were not statistically significant ($P = 0.064$), and they were not distinguishable from the average survival in the laboratory controls ($P = 0.067$), demonstrating that the unoiled sediments were not toxic.

Amendment of sediments with canola oil at both initial concentrations significantly reduced the survival of *H. azteca* in sediments that were not subjected to anaerobic biodegradation ($P < 0.002$), indicating that vegetable oil or its immediate degradation products were toxic to these benthic amphipods. After incubation for 2 weeks, during which anaerobic biodegradation of the vegetable oil occurred, survival in the sediments containing an initial oil concentration of 17 g/kg^{-1} was the same as the unoiled sediments ($P = 0.12$), demonstrating that the concentration of the toxic oil components or biodegradation intermediates were reduced to tolerable levels. Amphipod survival also increased in sediments containing a higher initial oil concentration (33 g/kg sediments) after 2 weeks of anaerobic biodegradation (54% ± 16%)

TABLE II. Average survival and average weight of *Hyalella azteca* in laboratory control sediments (mean ± standard deviation)

Time (weeks)	Survival (%)[a]	Weight (mg/specimen)[b]
0	96.7 ± 5.8	0.13 ± 0.02
2	96.7 ± 5.8	0.19 ± 0.04
8	100.0 ± 0.0	0.27 ± 0.01

[a] After 14-day exposure, the contents of each jar were sieved (0.5mm), and the survival was determined by observing mobile specimens under a dissecting microscope for 5–10 s; missing animals were assumed to be dead.

[b] The surviving specimens from each exposure beaker were pooled and dried at 60°C for 24 h then cooled for 1 h prior to determining the dry weight.

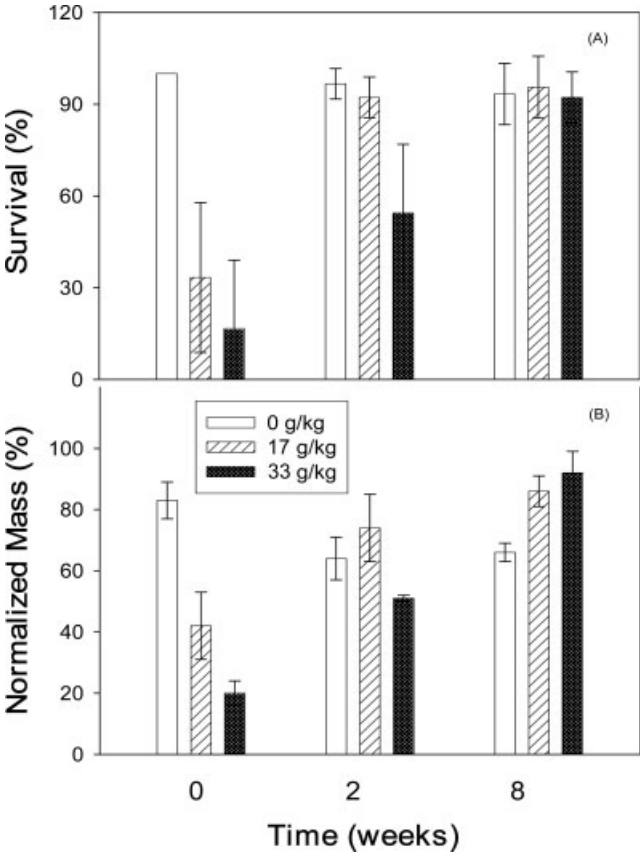

Fig. 3. Amphipod bioassays with *H. azteca*: (A) survival and (B) normalized dry mass of surviving animals following 14-day exposure.

compared to sediments that contained the same oil concentration but which were not incubated (17% ± 28%). After 8 weeks incubation, survival in all of the oiled sediments was the same as the oil-free control sediments ($P > 0.60$), indicating that the oiled sediments were no longer toxic by the criteria of *H. azteca* survival test.

In addition to survival, which is a measure of lethal toxicity, general health of the animals based on the growth of surviving amphipods was quantified to evaluate the sublethal toxicity of the oiled sediments over time. A significant increase in dry weight was observed in the laboratory control animals due to age differences (Table II), and therefore the weight of surviving animals in the unoiled and the oiled sediments were normalized by the weight of the animals in the laboratory control sediments at each time point to correct for batch-to-batch differences. Figure 3(b) shows the normalized dry mass of *H. azteca* after 14-day exposure to different treatments. At time zero, the average weight of the amphipods in the sediments containing canola oil at 17 and 33 g/kg was only 48 and 24%, respectively, of that in the unoiled sediments. Although the oiled sediments were initially toxic, the toxicity gradually decreased over time. After incubation for 2 weeks, the average weight of amphipods in the oiled sediments increased to nearly 115% (17 g oil/kg sediments) and 88% (33 g/kg) of the average weight of animals in the unoiled sediments. After 8 weeks of anaerobic degradation, the animals exposed to the oiled sediments were larger than those exposed to the unoiled sediments (probably due to increased nutrition in the sediments that contained oil), indicating that the oiled sediments no longer adversely affected the growth of the test animals. Therefore, like lethal toxicity, the sublethal toxicity of canola oil in sediments was completely removed during 8 weeks of anaerobic biodegradation.

CONCLUSIONS

It has already been shown that floating vegetable oil spills can be effectively removed from surface waters by sedimentation with clay minerals (Wincele et al., 2004), and the oil can be efficiently mineralized under anaerobic conditions in freshwater sediments (Li et al., 2001, 2005; Li and Wrenn, 2004). The objective of this study was to evaluate the toxicity of sediments contaminated by the concentrations of vegetable oil that can be produced by sedimentation of floating vegetable oil spills by clay and other dense minerals. The sediment toxicity was evaluated via two standard regulatory bioassays: the Microtox® SPT and a whole-sediment amphipod test using survival and growth endpoints.

The data presented here clearly showed that substances that inhibited the activity of *V. fischeri,* the test organism of the Microtox® SPT assay, were present during the early stage of anaerobic biodegradation of vegetable oil and that the inhibition decreased over time and was eventually eliminated after 8 weeks of incubation. Although the oil-contaminated sediments were inhibitory early in the oil degradation process, they would not be considered toxic based on regulatory threshold limits for contaminated sediments (Tay et al., 1997). The toxicity of the oil-containing sediments to the amphipod *H. azteca* was most severe immediately after addition of the vegetable oil (Fig. 3); the toxicity was significantly reduced after 2 weeks, and toxicity was not apparent after 8 weeks incubation. The toxicity encountered in the first 2 weeks of oil degradation was probably due to toxic intermediates (such as long-chain fatty acids), which are formed transiently during the anaerobic biodegradation of vegetable oil in freshwater sediments.

In summary, these bioassay results demonstrated that the toxicity of oil-contaminated sediments decreased over time as the oil degraded. While these laboratory findings may reduce concern over harmful effects of sedimented vegetable oil on benthic ecosystems, the results may not be directly transferable to all natural conditions. These results were obtained under somewhat idealized conditions, and any factors that reduce the rate of oil biodegradation would prolong the period of time during which the oil could adversely affect sensitive organisms. For example, the *in situ* temperature of sediments in deep lakes are typically much lower than the temperature used in the laboratory, and nutrient availability in the field could limit the oil biodegradation kinetics. Therefore, more comprehensive studies that evaluate the effects of temperature, nutrients, and other potentially limiting factors on the rates of production and removal of toxic intermediates are needed. In addition, chronic exposure tests involving a battery of test organisms covering several trophic levels are recommended to clearly define the long-term effects of sedimentation and biodegradation of vegetable oil on benthic ecosystems.

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